

Original Research Article

Studies on epidermal appendages of chick embryos

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ABSTRACT

Keywords

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beak;
Amino acids;
Keratin;
light;
Scanning and
transmission
Electron
microscopy.

The present study investigated the chicken skin appendages (abdominal feathers, beak and foot scales) at 9, 10, 12, 14 and 16 days old and revealed that keratin content is detected early in foot scales at 10-days embryo and then markedly detected at highest concentration in beak in developing embryos. The amino acid contents, especially serine, glutamine, proline, glycine, alanine, valine and leucine are detected at higher concentration in the different epidermal appendages. However the amino acids serine, glycine, alanine, leucine and isoleucine are more increased in foot scales more than the other body regions. SDS-PAGE of the studied regions exhibited marked keratin expression in foot scales more than dorsal body skin. Scanning electron microscopy (SEM) exhibited similar developmental origin. The feather filaments possess consequences of reconstruction with the formation of main axon and barb. The barbs characterized by its articulated cylindrical structural pattern. Dorsal body skin attained considerable thinning comparing with foot scales as well as lack of cornification. Different types of scales (reticulate, scutate and scutella) are detected during embryo development. TEM observation exhibited increased accumulation of keratohyaline granules of different forms in the cytoplasm of stratum granulosum of foot scales comparing with fine collection of keratin filaments in dorsal body skin.

Introduction

Cancer is the abnormal growth of cells in There are a variety of skin appendages, including feathers, scales, claws, and beaks in birds. Their morphogenesis involves the transformation of the primarily flat epidermis into specialized complex structures for induction, cell fate specification, proliferation, differentiation, epithelial cycling, and intimate

interactions with the mesenchyme (Chuong, 1998). Chicken skin offers distinct patterns of different cutaneous appendages, developed through consequences of epithelial-mesenchymal interactions (Choung and WidELITZ, 1998; Jiang *et al.*, 1998). Variations in developmental processes are thought to be

a key mechanism of organ novelty (Chuong, 1998).

The feathers of birds develop from embryonic epidermal lineages that differentiate during outgrowth of the feather germ. Scutate scales, which consist of peridermal layers, a subperiderm, and an alpha stratum. The feather-type B keratins are expressed in the subperiderm cells of embryonic scutate scales, as well as the barb ridge lineages of the feather. However, unlike the subperiderm of scales, which is lost at hatching, the cells of barb ridges, in conjunction with adjacent cell populations, give rise to the structural elements of the feather (Sawyer *et al.*, 2003a). From the dermomyotome, neural crest and somatopleura cells give rise to form the dermis. They interact with epithelium to form the skin and skin appendages. During these processes, regional specificities are endowed in development and evolution to generate diverse integuments and their appendages (Wu *et al.*, 2004). Jiang *et al.*, (2004) outlined several understand concerning how do these patterns form. Are they under strict genetic control? Then, why are many patterns similar but not identical?. Are they under epigenetic control? Then why do patterns appear to be amazingly consistent in animals of the same species?. The present study outlined the variations of developmental consequences of beak structure, feathers, foot scales using assaying of their contents of keratin and amino acids and protein electrophoresis. Light, scanning and transmission electron microscopy studies were carried out to clarify the diversity of their structural pattern.

Materials and Methods

One hundred fertile eggs from in crossbred

white leghorn hens were obtained from a commercial supplier. The eggs were incubated in a humidified incubator at $37 \pm 0.50^{\circ}\text{C}$, 60% relative humidity being turned three times daily. The chick embryos were collected at 9,10, 12, 14 and 16 days old according to Hamburger and Hamilton (1951).

Keratin content

Fresh specimens of skin were separated from the dorsum skin, foot and beak region and kept in nitrogen solution at -80°C . Keratin extractions were carried out at the selected 9,12 & 14-days old embryo according to Dreher *et al.*, (1998). An aliquot of 1 ml of 1 M NaOH was added to a 2 ml cryo vial with the rolled tape strip, and the tube was stored for at 4°C overnight. The next morning, 1 ml of 1 M HCl was added and the sample and mixed thoroughly. A 100 μl aliquot of skin sample was aliquoted with 100 μl of 1 M NaCl and one milliliter of Bradford 's reagent and allowed to stand at room temperature for 2 min before measuring absorbance at 595 nm using a UV spectrophotometer (Beckman DU640; Beckman Instruments, Palo Alto, CA). A standard curve prepared to use commercially available human keratin (Sigma, St Louis, MO) and determination of keratin was carried out.

Amino acid analysis

The dorsal body skin, foot scales and beak of 16 days old embryos were hydrolysed by 6M hydrochloric acid. of the selected specimens were dissected and hydrolysed by 6M hydrochloric acid. Sensitive amino acids (especially Tryptophane and cysteine) will be partially destroyed. The samples were washed in hot dilute detergent solution at neutral pH and rinsed

in warm tap water and then distilled water. Any pulpy protein in the column was squeezed out and extracted several times with petroleum ether, followed by 95% ethyle alcohol and allowed to dry in a watch glass. The samples were dried under vacuum, redissolved in 10 to 100 μ l 0.2 M sodium citrate buffer, pH 2.0, and loaded on the amino acid analyzer equipped with a cation exchange column (Amersham Pharmacia Biotech), which was equilibrated in 0.2 M sodium citrate buffer, pH 2.0. Elution was performed with a gradient of pH and ionic strength as instructed by the manufacturer. Detection of the modified amino acids was achieved calorimetrically at 440 nm for proline and hydroxyproline and at 570 nm for all other amino acids (Niece *et al.*, 1991).

SDS-PAGE analysis of keratin and protein

Fresh biopsies of skin, foot scales and beak at 9, 12 & 14-days old embryo were examined by sodium dodecylsulfate polymerase gel electrophoresis according to the method of Laemmli (1970). Electrophoresis was carried out at a constant 200 V. The separated proteins were placed on polyacrylamide gels stained with Coomassie blue R-250 (60 mg/L) in an acidic medium for the generation of an electrostatic attraction between the dye molecules and the amino groups of proteins (Andrews, 1986). In case of keratin electrophoresis, two kinds with different molecular weight were used as standard against the extracted protein from the investigated specimens.

Light microscopic investigation

Dorsal body skin, foot scales and beak region of 9, 12 & 14-days old chick

embryos were incised immediately at developing stages and fixed in 10 % formal saline and processed for histological investigation and examined, photographed under bright field light microscopy.

Scanning electron microscopic investigation

Dorsal body skin and foot scales of developing chick embryos were separated and immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After washing the samples with deionized water, they were stepwise dehydrated in 50% (v/v), 80% (v/v), absolute ethanol. Drying was done in a carbon dioxide critical point drying apparatus. The samples were mounted on aluminum stubs, coated with a thin layer of gold by low voltage DC sputtering and viewed using a Joel 5300JSM (Musashino 3-chome Akishima Tokyo 196-8558, Japan).

Transmission electron microscopic investigation

Dorsal body skin and foot scales of developing chick embryos were separated and immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After rinsing in 0.1 M cacodylate buffer, they were post fixed in a buffered solution of 1% osmium tetra oxide at 4°C for 1.5 hour and dehydrated in ascending grades of ethyl alcohol and embedded in epoxy-resin. Ultrathin sections were cut with a diamond knife on a LKB Ultratome IV ((LKB Instruments, Bromma, Sweden) and mounted on grids, stained with uranyl acetate and lead citrate, and examined under a joel 100CX transmission electron

microscope (Musashino 3-chome Akishima Tokyo 196-8558, Japan).

Results and Discussion

Keratin and amino acids content

Figure (1,2) illustrated the keratin and amino acids contents in dorsal feathery skin, foot scales and beak respectively. The keratin is markedly increased in foot scales of 10-days old embryo comparing with feathery skin and beak region. At 12 and 14-days old embryo, the keratin attained highest increase in the beak region. The amino acid contents of serine, glutamine, proline, glycine, alanine, valine and leucine are detected at higher concentration more than the other estimated amino acids and varied markedly between the mentioned regions. The amino acids serine, glycine, alanine, leucine and isoleucine are markedly increased in foot scales more than the other body regions. There are least variations of the others amino acid content in feathery skin, foot scales and beak regions.

Scanning, light and transmission electron microscopy of feathers

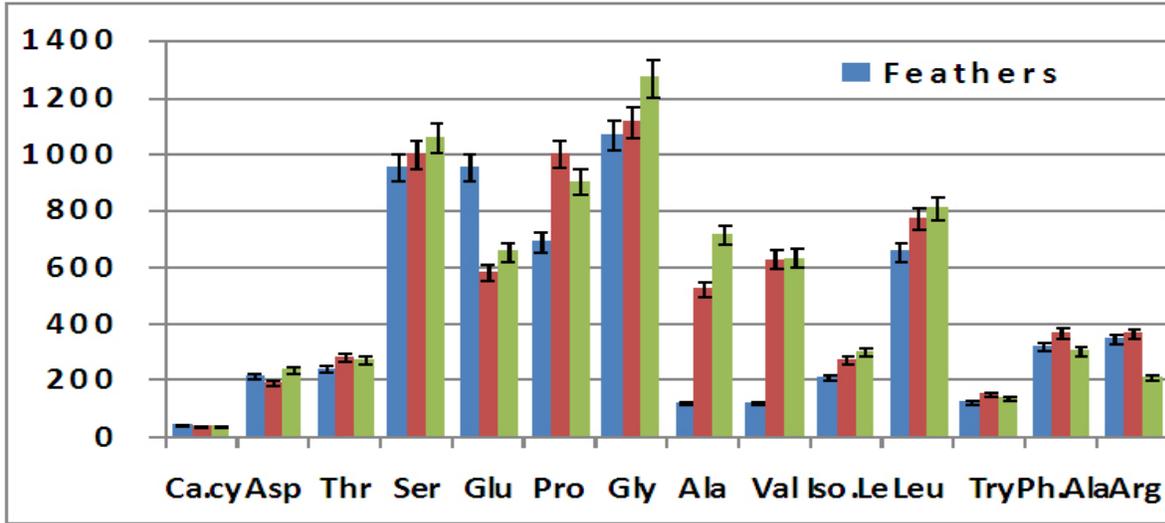
Scanning electron microscopic observations of nine-day old embryo revealed that, the feather buds primordia forming apical ectodermal ridge as a result of epidermal - dermal condensation. (Figure. 3A). The feathers take cylindrical pattern structure at 12 day-old chick embryo (Figure. 3B). At 14 days-old chick embryo, the feather follicles possess necrotic zones forming barbs at regular spaces. The base of each feather filament invaginated into the dermis forming feather-epidermal collar at feather epidermal junction. The terminal end of the feather takes the criteria of feather

cone apex. The barbs appear intermingled with each others (Figure. 3C). At 16 days-old, the feather proper structure is detected. It is composed of a main shaft from which emerged barbs and barbules. The barbs and barbules are formed of cylindrical articulated structures (Figures 3D-F).

At the light microscopic level, the feather follicle starts in the form of a placode of epidermal-dermal condensations at 9-days old. Fibroblasts are more dense in the dermis and infiltrated in between the collagen fibrils and blood vessels (Figures 4A). The morphogenesis of follicles proceeds in the subsequent stages 10 and 12-days old embryo. The feathers elongated in proximal-distal axis and cross sections possess regularly oriented barb plates at different levels of the ramus (Figures 4 B-D).

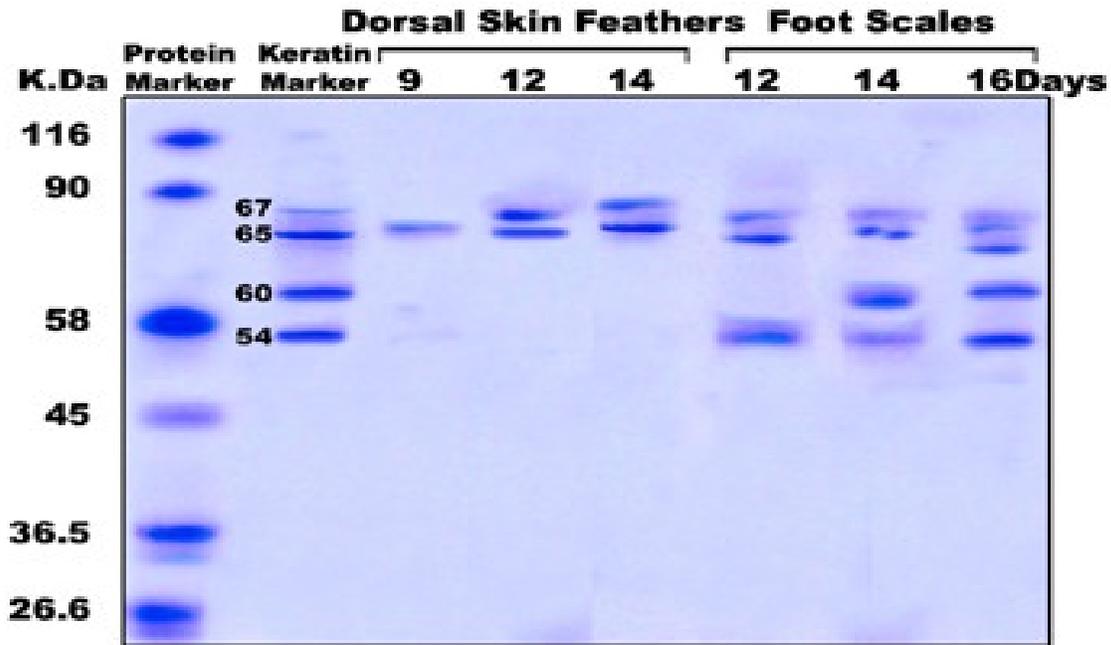
At transmission electron microscopic level, 11-days old embryo possessed thinned epidermis composed of two-cell layers thick. The innermost cell layer is stratum germinativum composed of more active oval-shaped cells. Their cytoplasm showed abundant rough endoplasmic reticulum, mitochondria and free ribosomes. The epidermal-dermal junction is irregular. The dermis is enclosed with fibroblast cells. The other kinds of cells are peripherally located and lacked the presence of keratohyalin granules and similar to stratum spinosum cells (Figures 6 A&B). At 14-days old embryo, the epidermis is composed of four cell layers thick. Both epidermal cells exhibited abundant distribution of mitochondria, rough endoplasmic reticulum and free ribosomes in their cytoplasm. The peripheral cell layer suspected to be stratum granulosum cells as a result of cytoplasmic distribution of fine keratohyaline granules (Figures 6 C,D).

Figure.1 Amino acids contents of feathers, beak and foot scales of 16 days chick embryo.



Ca.cy, cysteine; Asp, Asparagine; Thr, Threonine; Ser, Serine; Glu, Glutamine; Pro, Prolline; Gly, Glycine; Ala, Alanine; Val, Valine; Iso.Le, Isoleucine; Leu, Leucine; Try, tyrosine; Ph.ala, Phenylalanine; Arg., Arginine. Each result represents the mean ± standard error of five replicates.

Figure.2 SDS-PAGE of keratin analysis in dorsal skin of foot scale of developing chick embryos.



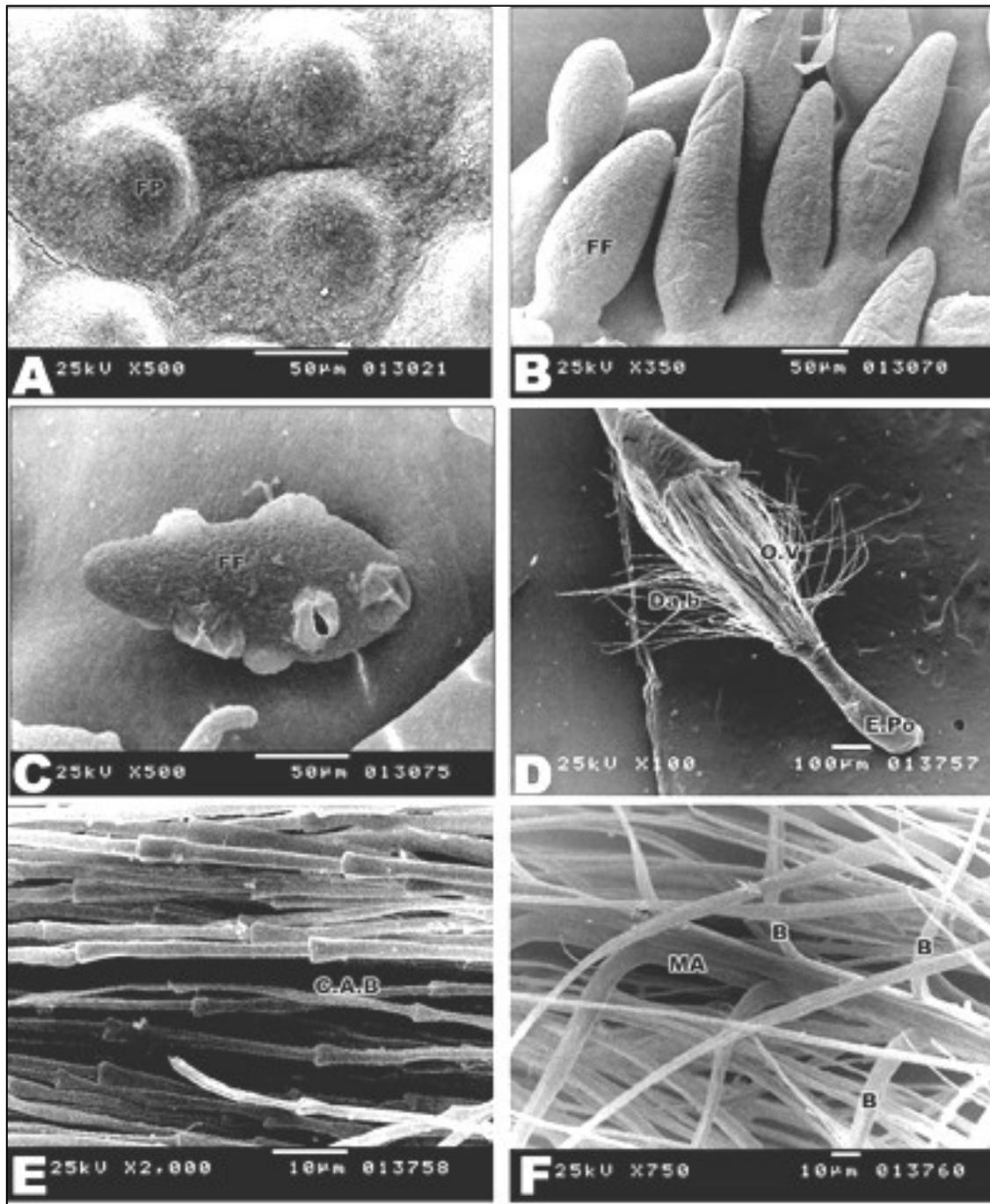


Figure.3 (A-F) Electron micrographs of developing feathers of chick embryos. A.Nine-days old. B. Twelve-days old. C. Fourteen-days old. D. E & F. Sixteen-days old. (Abbreviations: B, Barb, CAB, Cylindrical articulated barb; MA, Main axon; FF, Feather filament; FP, Feather papilla; OV, Opened vans).

Scanning, light and transmission electron microscopy of foot scales

Scanning electron microscopy exhibited SEM level, more organized foot plate in the nine-days old embryo. The epidermis formed the placode and interplacode cell populations. The primordial structures of the scutate scales are detected (Figures 5A, B). At the 12-days old embryo, morphogenesis of scutate scales appeared in the form of folded ridges over the toes surface. The scutate scales are large and rectangular in shape and overlapping with each other. Cornification of the claw is proceeded (Figures 5C-F). At the 14-days old embryo, the dorsal foot surface is composed of overlapping scutate scales. The scutella scales are distributed lateral to the scutate scales and are smaller in size. The third types, reticulate scales are detected on the foot pad and characterized by their radial symmetry (Figures 5 G-I) . At the 16-days old embryo, the different parts of the tarsal shank are covered with several sheets of cornification (Figures 5 J, K).

At the Light microscopic level, 12-days old chick embryo, foot region exhibited different pattern structure of epidermal scales. Scutate scales are the largest form and have both similar thickness of their apex and hinge region formed of two-cell layers thick. Scutella represents the second types of scales and is detected in the ventral surface. The scales appeared more flatter and regular oriented but lack overlapping pattern structure as scutate scales. A third type is reticulate scale in the lateral surface. (Figures 4 A1,B1). At 14 and 16-days old embryo, keratinization of the epidermis of the different types of scales is detected. (Figures 4 C1-E1).

Ultrastructural observations of scales of 14

days-old embryo revealed that the epidermis is composed of several cell layers thick. The stratum germinativum possessed characteristic hemidesmosomes infiltrated their parietal surfaces. The stratum spinosum cell oval-shaped and has centrally located nuclei with two or three nucleoli. The intercellular space is enclosed by threads of keratohyaline granules.

Toward the peripheral surface, the stratum granulosum is more recognized with peculiar large keratinocytes having indented nuclear envelope rich of euchromatin and containing one nucleoli. Their cytoplasm as well as the intercellular space is rich in keratohyaline granules of both vesicular characteristic structure and varying sizes. Cornification of the outer surface is more detected. The keratinized layers are composed of several layers and enclosed by newly formed and dead corneocytes. The epidermal dermal junction is markedly folded. The dermis possesses abundant distribution of collagenous fibrils, fibroblasts and blood vessel (Figures 6 A1-D1).

During development of chick embryos, the integument possessed diversity of epidermal structures in abdomen, foot and beak region. Although they are markedly different, they share common developmental pathways. The observed findings of skin development of both abdomen and foot region revealed that they showed the regular sequence of growth pattern, including the formation of a placode stage involving sequences of invagination in the dermis at nine days. However, with the subsequent developing ages, the growth patterns are markedly deviated. The feather germs grow in a proximal-distal axis forming a tube with

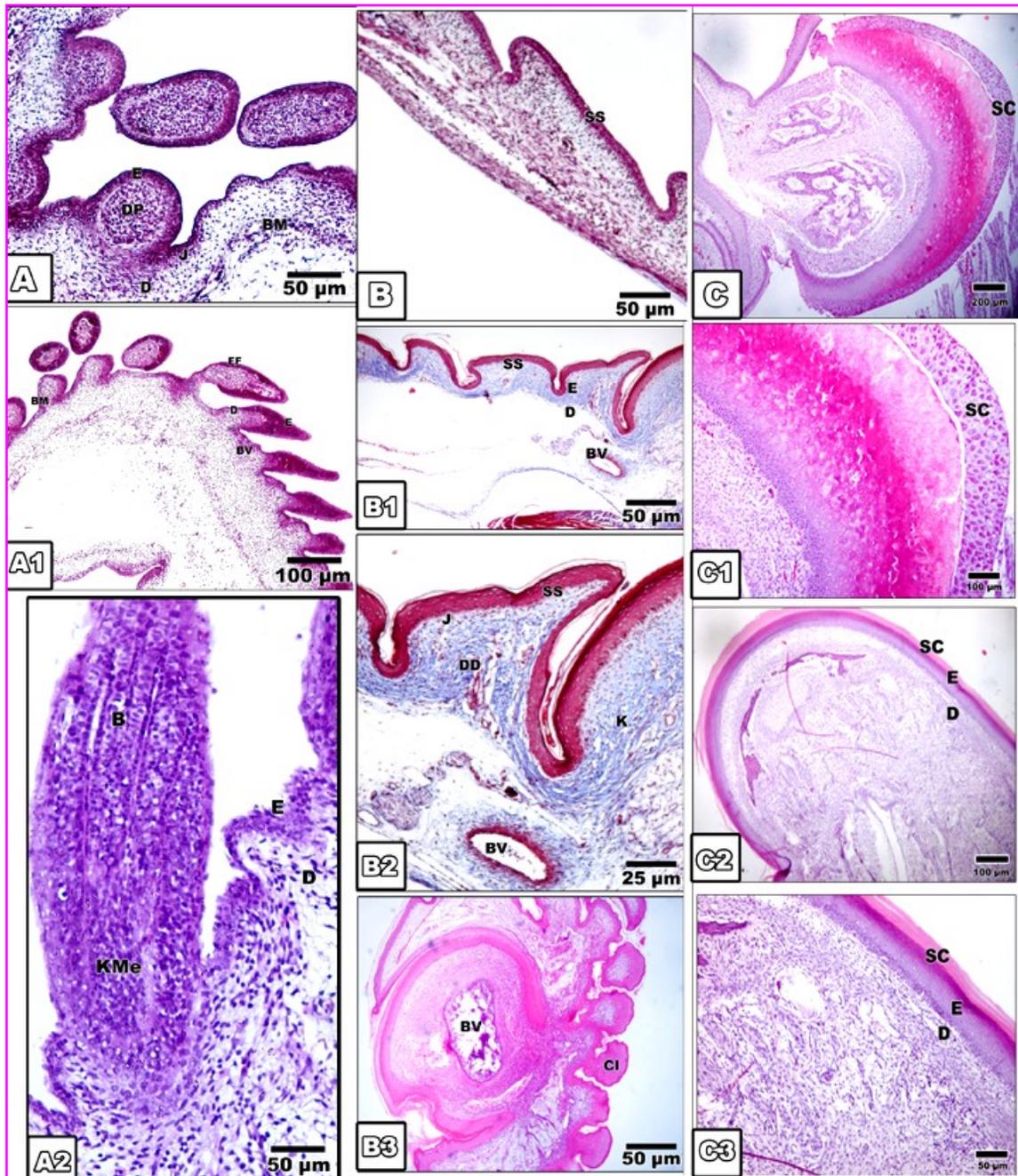


Figure.4(A-C3) Histological micrographs of feather (A-A2), foot scales (B-B3) and beak of chick embryo.

A. Feather histogenesis of 9-days old. A-A2. Feather histogenesis of 10 days-old. B. Foot scale histogenesis of 9 days old embryo B1-B3. Foot scale histogenesis of 16 days old embryo. C-C1. Beak cornification of 9 days old embryo. C2C3. Beak cornification of 10 days old embryo (Abbreviations: B, Barbs; C, Cornification; D, Dermis; R, Reticulate scale; SC, stratum corneum; SS, Scutate scale; Sc, Scutella).

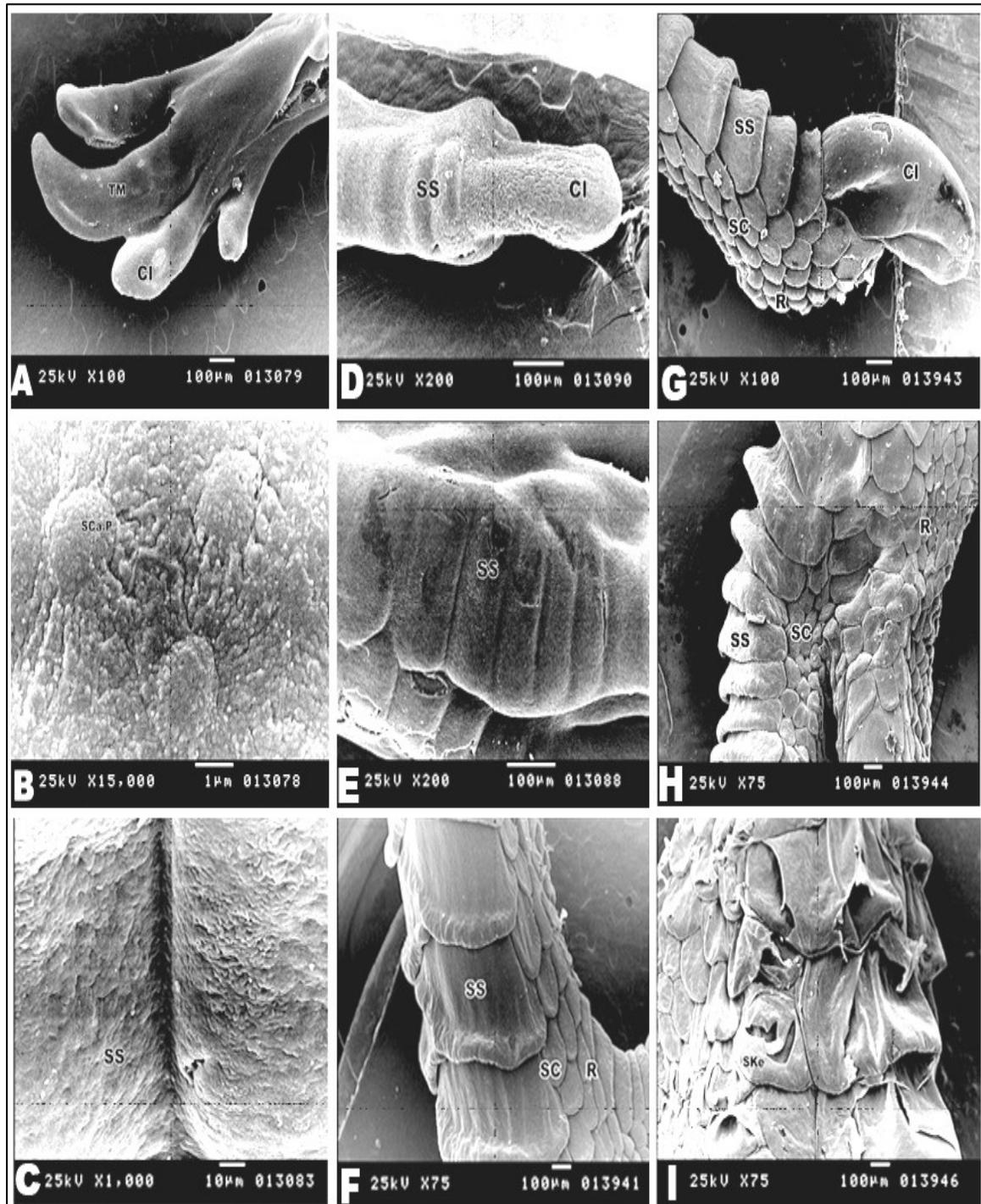
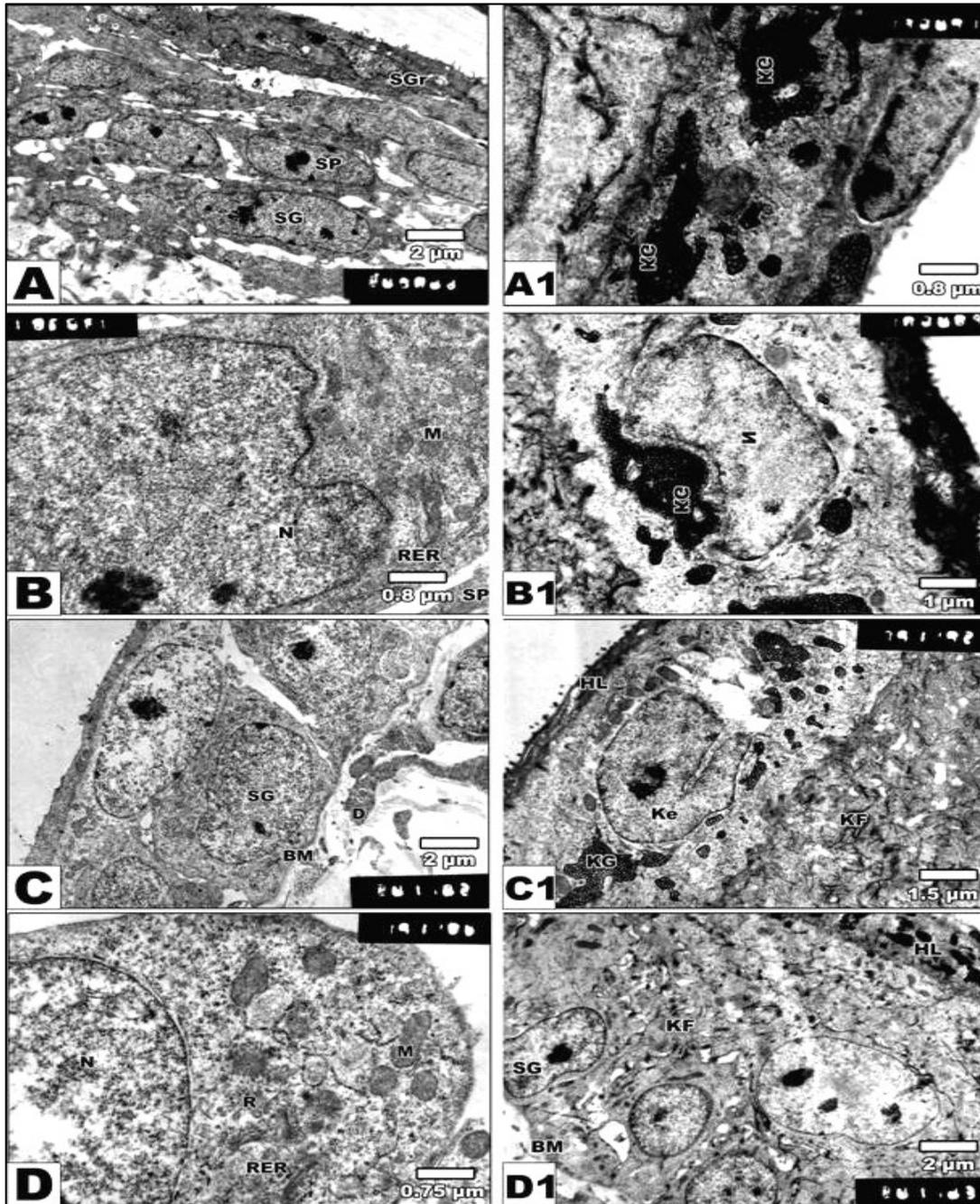


Figure.5(A-I) Electron micrographs of foot scales of developing chick embryo. A&B. Nine-days old. C- E. Twelve-days old embryo. F-H. Fourteen-days old. I. Sixteen-days old.(Abbreviations: CI, Claw; R, Reticulate scale; SS, Scutate scale; Sc, Scutella; SKs, Shedding keratin).

Figure.6 (A-D& A1-D1) Electron micrographs of dorsal body skin (A-D) and foot scales (A1- E1). A& B.Epidermal cell layers of 11 days old embryo. C & D. epidermal cell layer of 12 days old embryo. A1-D1.Epidermal cell layers of foot scales of 14 days old embryo.



Note: Keratinocytes and cytoplasmic inclusions of keratohyaline granules. (Abbreviations: KF, keratin Filament; KG, Keratohyaline granules; M, Mitochondria; N, Nucleus; RER, rough endoplasmic reticulum; SG, Stratum germinativum; SGr, Stratum granulosum; SS, Stratum spinosum).

early origin of barbs followed by regularly oriented parietal surface of the follicle tube at 10 & 12-days old. The epidermis is differentiated into two or three cell layers thick having the innermost germinativum cells, and the outermost is the perspective keratinocytes. Cornification is less detected in the epidermis of skin comparing with that of the feather. Also, the epidermal placode of foot is further developed into three kinds of scales scutate, scutella and reticulate scales. The beak shows another pattern of developmental programmes, employing epithelial and mesenchymal cells to produce composite structures, forming the architecture structure specific of chick with a glance cornification of its outer covering sheath. The present findings agree with the findings of Dhouailly (1978), Wu *et al.*, (2004), Schneider (2005) and Widelitz *et al.*, (2006).

According to Webb and Noden (1993) and Schneider (2005), the variations of beak structure from feathers started from earlier embryonic development and formation of primordial tissue. The upper beak region is derived from the frontonasal and paired maxillary primordia, whereas the lower portion forms from paired mandibular primordia. There was a patterning potential of cranial neural crest mesenchyme during beak morphogenesis (Schneider and Helms, 2003).

SEM observations of feather and scale development during embryonic development reflected the closely homologous of their early cone differentiation pattern; however, diversity occurred of their initial formation. Feathers primordia showed a similar characteristic structure in light microscopic level, however, striking findings at the 14-days old, the feather proper tubular structure is composed of the

main shaft from which emerged barbs and barbules. The barbs and barbules appeared in the form of cylindrical articulated structures. The structural form of avian scales comprised three main types, scutate, scutella and reticulates. They composed mainly of almost similar thickness of their outer scale surface and hinge region reflected absence of articulation of the scales as in reptilian section. The epidermis is composed of several cell layers thick with the marked degree of cornification being at the highest grade in the beak region. The dermis is denser of fibroblast and collagen fibrils.

The present findings supported the work of O'Guin *et al.*, (1982), Haake *et al.*, (1984) and Haake, (1985). According to Fisher *et al.*, (1988), Chuong (1998) and Chuong *et al.* (2003), despite diversity of epidermal appendages, they have many similar developmental origins.

In addition, we observed marked variations of epidermal thickness between abdominal skin feathers, foot skin and beak region, being more thickened in beak and foot skin. The peripheral cell layers of foot regions characterized by increase of keratinocytes and keratohyaline granules of different size. The beak epidermis becomes more cornified with characteristic glossy appearance acquiring the structural appearance of the beak required for feeding habit.

Also, we detected that the biochemical and molecular analysis of keratin, amino acids and protein expression confirmed variations of keratinization of the epidermal appendages. Foot scales exhibited highest keratin formation at 10-day old embryos in comparison with abdominal skin and beak regions. However, keratin content attained more

increase in the beak region at 12 and 14-day old embryo. The amino acid contents of serine, glutamine, proline, glycine, alanine, valine and leucine are detected at higher concentration in foot scales more than that of other body regions. Although there is a relative similar arrangement of amino acids, their concentration varied according to the degree of cornification in different epidermal regions, which is parallel with the function of developmental part either in feeding for beak or protection from damage as in the foot region.

According to recent molecular biology and proteomic studies carried out by Dalla Valle *et al.*, (2005, 2007), the highest concentrations of serine, glutamine, proline, glycine, alanine, valine and leucine reflected the degree of keratin formation. The homology of the core-box among different epidermal appendages species suggested that this amino acid sequence and concentrations evolve from a progenitor sequence present in the stem of avian skin. The core-box of amino acids is implicated in the formation of keratin filaments of scales, claws, and feathers (Gregg and Rogers, 1986; Fraser and Parry, 1996).

Finally, the authors concluded that, the integument structures have close similarities in their early origin but markedly different in their initial destination in both fine structures and their keratin and amino acids contents.

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